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Immunology

Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein GP96/GRP94

(heat shock protein/tumor vaccine)

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ABSTRACT **Heat shock protein gp96 primes class I restricted cytotoxic T cells against antigens present in the cells from which it was isolated. Moreover, gp96 derived from certain tumors functions as an effective vaccine, causing complete tumor regressions in *in vivo* tumor challenge protocols. Because tumor-derived gp96 did not differ from gp96 isolated from normal tissues, a role for gp96 as a peptide carrier has been proposed. To test this hypothesis, we analyzed whether such an association of antigenic peptides with gp96 occurs in a well-defined viral model system. Here we present the full characterization of an antigenic peptide that endogenously associates with the stress protein gp96 in cells infected with vesicular stomatitis virus (VSV). This peptide is identical to the immunodominant peptide of VSV, which is also naturally presented by H-2K^b major histocompatibility complex class I molecules. This peptide associates with gp96 in VSV-infected cells regardless of the major histocompatibility complex haplotype of the cell. Our observations provide a biochemical basis for the vaccine function of gp96.**

gp96, also known as GRP94, is a member of the HSP90 family of stress proteins that is present in the endoplasmic reticulum (ER) (1, 2). The protein is targeted to the ER by an amino-terminal signal sequence and contains the KDEL amino acid motif responsible for its retention in the ER lumen (2, 3). There is some biochemical evidence suggesting that gp96 can also exist as a transmembrane molecule (4–6). The expression of gp96 is up-regulated by stress conditions that result in the accumulation of unfolded proteins in the ER (7). gp96 has been found to associate with unassembled immunoglobulin chains, major histocompatibility complex (MHC) class II molecules, and a mutant glycoprotein B from herpes simplex virus (8–11). Based on these observations, it was suggested that gp96 might be a chaperone assisting folding/association of such multi-subunit proteins in the ER, analogous to its family member Hsp90, which has a similar function in the cytosol (12).

Recently, it has been observed that gp96 molecules purified from chemically induced tumors efficiently function as tumor vaccines, causing complete tumor regressions in *in vivo* tumor challenge protocols (13–15). This tumor-protective effect cannot be ascribed to changes in the gp96 molecules themselves, because tumor-derived gp96 did not differ from gp96 of healthy tissues (16). Together with the fact that CD8$^+$ T cells play a role in the observed tumor regression (17), these observations lead to the hypothesis that short peptides associating with gp96 may be the antigenic entities responsible for the protective effect of tumor-derived gp96 (18). Because no antigens have been characterized in the tumor models used in the *in vivo* protection studies, this hypothesis has yet to be formally proven. Therefore, we analyzed whether association of antigenic peptide(s) with gp96 molecules occurs in a well-defined viral model system.

MATERIALS AND METHODS

Cells. EL4 and RMA-S are H-2b T-cell lymphomas, and N1 cells are EL4 cells transfected with the gene that encodes the nucleocapsid protein of vesicular stomatitis virus (VSV). P815 is an H-2d mastocytoma. The cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO/BRL), supplemented with 5% fetal calf serum, 20 μM 2-mercaptoethanol, and antibiotics. VSV-specific cytolytic T lymphocyte (CTL) clones N15, N20, N26, N32, and N39 (kindly provided by S. G. Nathenson, Albert Einstein College of Medicine, Bronx, NY) have been described in detail (19, 20). The clones are all specific for the immunodominant peptide VSV8 (corresponding to amino acid residues 52–59 of VSV nucleoprotein) in the context of H-2K^b molecules, but all use a unique set of antigen-specific T-cell receptor (TCR) α,β-chains, resulting in different fine specificities. The clones were maintained by weekly stimulations with 0.5×10^6 irradiated [20,000 rad (1 rad = 0.01 Gy)] N1 cells, 4×10^6 irradiated (3,000 rad) C57BL/6 (B6) spleen cells per 0.2×10^6 CTL, in 2 ml IMDM supplemented with 10% fetal calf serum, 2-mercaptoethanol, antibiotics, and 3% culture supernatant of concanavalin A-stimulated blast cells of rat splenocytes.

Purification of gp96 and H-2K^b. Cells ($2–5 \times 10^9$) were infected with 10 plaque forming units (pfu) per cell of VSV, Indiana strain. After 8 h of infection, cells were harvested and washed twice in PBS (pH 7.4). From these cells, as well as from equal numbers of uninfected cells, ER luminal gp96 was isolated as described by Udono and coworkers (17) but adapted to scale. Briefly, cell pellets were dounce homogenized in four volumes of hypotonic buffer (30 mM $NaHCO_3$/0.5 mM phenylmethylsulfonyl fluoride, pH 7.1) and centrifugated for 10 min at $4,500 \times g$ and then at $100,000 \times g$ to remove cell membranes and nuclei. The $100,000 \times g$ supernatant was applied to ConA Sepharose (0.5-ml bed volume/ml of original cell pellet). ConA-bound material was eluted with PBS containing 1 mM $MgCl_2$, 2 mM KCl, and 10% α-methylmannopyranoside. This material was separated by fast protein liquid chromatography using a Mono Q column (HR 5/5; Pharmacia) with a 0-1 M NaCl gradient. Fractions (1 ml) were collected. Aliquots (1 μl) of each fraction were loaded onto SDS/10% polyacrylamide gels, and gp96 was identified after

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Abbreviations: ER, endoplasmic reticulum; MHC, major histocompatibility complex; VSV, vesicular stomatitis virus; CTL, cytolytic T lymphocyte; TCR, T-cell receptor; TAP, transporter associated with antigen processing.

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Western blotting using anti-gp96 antibody SPA 850 (clone 9G10; StressGen Biotechnologies, Victoria, Canada). Fractions containing single gp96 bands on silverstained gels were used in experiments.

Combined membrane fractions after the two centrifugation steps were pooled and used to immunoprecipitate H-2K^b molecules. Membrane fractions were solubilized in 0.5% Nonidet P-40 in 20 mM Tris·HCl (pH 7.5) and 150 mM NaCl. H-2K^b molecules were precipitated as described (21) using rabbit anti-P8 serum [recognizing the cytoplasmic tail of H-2K^b; kindly provided by H. L. Ploegh (22)].

Peptide Stripping from Cell Surface MHC Class I Molecules. EL4 cells (5×10^7) and P815 cells (10^8) were infected with 10 pfu of VSV per cell. After 8 h, cells were thoroughly washed in PBS. Then, peptides associated with surface MHC class I molecules were acid eluted by 0.65% trifluoroacetic acid (TFA) as described by Franksson and coworkers (23), resulting in final volumes of 1 ml. The pH of the peptide pools was adjusted to 7.5. Peptides (25-μl aliquots) were tested in triplicate for their ability to sensitize EL4 cells (2000 per well) for recognition by clone N15 in a standard ^{51}Cr release assay (21).

Peptide Isolations. Peptides were stripped from gp96 and H-2K^b molecules by acid elution (500 μl of 0.05% TFA). The low-molecular-weight fractions were separated from high-molecular-weight material by centricon centrifugation (3-kDa cutoff; Amicon). This procedure was repeated once, resulting in two fractions that were combined, dried, redissolved in PBS, and pH adjusted to 7.5.

HPLC Fractionation. Bulk peptide fractions isolated from gp96 and H-2K^b molecules were fractionated by reverse-phase chromatography on a Pharmacia C2/C18 2.1/10 column on a SMART microanalytical HPLC system (Pharmacia). The gradient was made of solution A (0.1% TFA in H_2O) and solution B (0.875% TFA in acetonetrile). During a linear gradient of 10–60% of solution B, a total of 60 fractions (100 μl per fraction) were collected. These fractions were reduced in volume by speedvac and redissolved in 200 μl IMDM.

CTL Assays. Bulk peptides isolated from gp96 and H-2K^b (purified from 5×10^9 EL4 cells, 150-μl volumes corresponding to 1/4 of total isolated material) were incubated for 30 min at room temperature with 1.5×10^6 RMA-S target cells, which had been preincubated overnight at 26°C to up-regulate MHC class I molecules. Subsequently, the cells were labeled for 1 h with 100 μCi (1 Ci = 37 GBq) of ^{51}Cr. The presence of antigenic materials was analyzed in a standard chromium release assay using VSV-specific CTL clones. When HPLC fractions were tested in chromium release assays, the fractions were dried using speedvac centrifugation redissolved in 200 μl of PBS, and 20-μl aliquots were added to triplicate wells of 96-well plates, containing 2000 target cells. The presence of antigenic materials was analyzed in a standard 4-h chromium release assay using VSV-specific CTL clones.

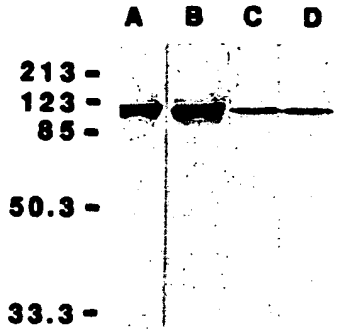

FIG. 1. SDS/PAGE analysis of purified gp96. gp96 isolated from 5×10^9 VSV-infected EL4 cells (lane A) and 5×10^9 uninfected EL4 cells (lane B) was identified in fractions collected after Mono Q separations on Western blots. Fractions containing single gp96 bands on silverstained gels (lane C, VSV-infected EL4 cells, and lane D, uninfected EL4 cells) were used in peptide stripping experiments.

RESULTS AND DISCUSSION

We used VSV-infected cells as a model system to study the association of peptides with gp96. In previous studies, we established that the CTL response against VSV in H-2b mice is directed against a single octamer peptide VSV8 (RGYVYQGL, amino acid residues 52–59 of the nucleoprotein), which is presented on the surface of infected cells by the MHC class I molecule H-2K^b (21). Two batches of EL4 cells (H-2b), one infected with VSV and one uninfected, were used to purify gp96 molecules. As a control for adequate virus infection and proper antigen processing, the presence of VSV8 in association with H-2K^b molecules was analyzed in the same cell preparations. The purity of the gp96 preparations was checked by SDS/PAGE, and gp96 was identified by Western blotting (Fig. 1). A single band was detected in both gp96 preparations (Fig. 1, lanes C and D). Low-molecular-weight materials associating with these gp96 molecules and with H-2K^b molecules were isolated by acid treatment, followed by filtration over centricon filters. Strikingly, in the total peptide mixture that associates with gp96 molecules in VSV-infected EL4 cells, the presence of peptide(s) able to sensitize RMA-S cells (H-2b) for recognition by five VSV8-specific CTL clones could easily be demonstrated (Table 1). In peptide mixtures isolated from gp96 of uninfected EL4 cells, no antigenic activity was detected (Table 1). The fine specificity of the clones used has previously been characterized in detail (19). Each clone expresses a unique pair of TCR α,β-chains and recognizes the K^b/peptide surface facing the TCR with different fine specificity.

Table 1. The peptide mixture stripped from gp96 of cells infected with VSV sensitizes target cells for recognition by virus-specific CTL clones

	Specific lysis of RMA-S target cells, %					
			Bulk peptides stripped from EL4			
CTL clone	+VSV8*	−VSV8†	+VSV K^b	−VSV K^b	+VSV gp96	−VSV gp96
N15	74	4	68	3	47	3
N20	73	5	65	3	45	0
N26	80	4	74	5	45	0
N32	70	29	61	15	47	12
N39	73	6	63	0	50	0

gp96 molecules were isolated from EL4 cells infected with VSV and uninfected EL4 cells as described in the legend to Fig. 1. The membrane fractions of both cell populations were used to immunoprecipitate H-2K^b molecules.

*Synthetic VSV8 (150 μl at 400 μM in PBS) was used as a positive control.

†Negative control, PBS. Percent specific lysis of 3:1 effector to target ratios are shown as a mean of triplicates.

The five clones are highly sensitive to defined, single amino acid substitutions and slight changes in the three-dimensional orientation of amino acid side chains (pointing toward the TCR) in the peptide (19, 20). Thus, the observation that all clones recognize the bulk of peptides associating with gp96 establishes the presence of peptide(s) containing the amino acid sequence of VSV8 as a core.

To further characterize the viral antigenic peptide material that endogenously associates with gp96, we separated the peptide mixture by HPLC. The separation profile was simultaneously monitored at 214, 254, and 280 nm, and fractions were collected. Comparison of the HPLC profiles of VSV-infected and uninfected EL4 cells did not reveal peaks specific for virus-derived material (data not shown). Using the more sensitive *in vitro* CTL assay, we identified an HPLC fraction that could sensitize H-2b-positive target cells (Fig. 2*A*). This positive fraction was found at the exact same acetonitrile concentration as the immunodominant peptide that is naturally associated with K^b molecules (Fig. 2*B*) and was previously identified as VSV8 (ref. 21; Fig. 2*C*). Importantly, in HPLC fraction 19 of gp96-associated material, a peptide with a mass of 956 Da could be identified as the major peptide by mass spectrometry (Fig. 3*A*); this mass exactly equals the mass of VSV8 (Fig. 3*B*). In the corresponding HPLC fraction of uninfected EL4 cells, this mass was not detected (Fig. 3*C*).

Because gp96 has been proposed to function as a chaperone in the assembly of protein complexes, we next addressed the risk that traces of MHC class I, copurifying with gp96, were the source of immunogenic peptides. Therefore, we determined whether the association of VSV8 with gp96 depends on the simultaneous presence of K^b molecules in the cell. We purified gp96 molecules from VSV-infected and uninfected P815 cells (H-2d). Because VSV8 does not associate with H-2d class I molecules in VSV-infected P815 cells (Table 2), any peptide

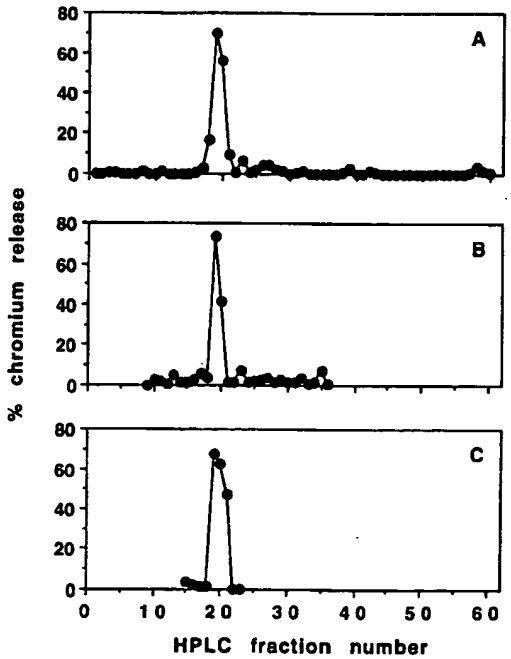

FIG. 2. Antigenic material eluted from gp96 comigrates on HPLC with VSV8. Half of each peptide fraction isolated from gp96 and K^b molecules purified from VSV-infected EL4 cells (described in Table 1) was fractionated by reverse-phase chromatography. Collected fractions were tested for their ability to sensitize EL4 cells for recognition by CTL clone N15. The percentage-specific lysis at a 5:1 effector to target cell ratio is plotted vs. the fraction number. (*A*) Peptides stripped from gp96. (*B*) Peptides stripped from K^b. (*C*) Synthetic peptide VSV8.

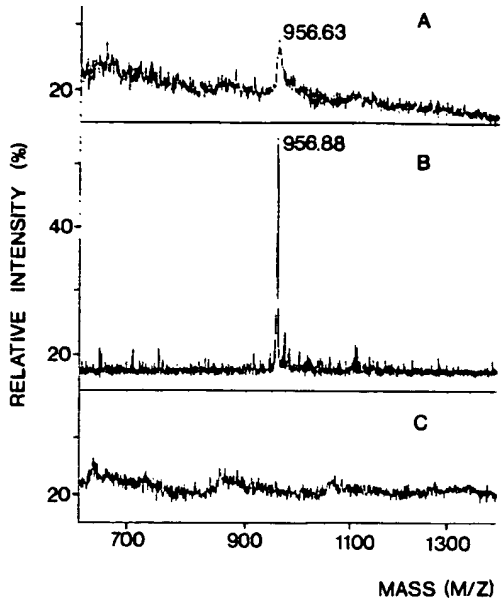

FIG. 3. VSV8 is identified as the antigenic peptide endogenously bound to gp96 in VSV-infected EL4 cells. Using Matrix-assisted laser desorption time-of-flight mass spectrometry (Lasermat, Finnigan, U.K.), a single peptide of 956-Da was identified in HPLC fraction 19 of gp96 (Fig. 2*A*) eluted material (*A*). This equals the mass of synthetic VSV8 (*B*). In the corresponding HPLC fraction of uninfected EL4 cells, this mass was not detected (*C*).

recovered from gp96 from VSV-infected P815 cannot be derived from contaminating traces of MHC class I molecules. An antigenic peptide eluting on HPLC at the same acetonitrile concentration as VSV8 could be detected in peptide mixtures associating with gp96 molecules in VSV-infected P815 cells but not in uninfected P815 cells (Fig. 4). Thus, the MHC haplotype of cells does not influence the binding of peptides with gp96. The finding that VSV8 is also recovered from gp96 preparations produced from H-2d-positive cells formally demonstrates that the association of VSV8 with gp96 is due to a direct gp96–peptide interaction and not caused by contaminating traces of MHC class I molecules.

VSV8 has been described as the sole immunodominant peptide recognized by bulk CTL against VSV in C57BL/6 mice (21). However, other viral peptides may associate intracellularly with gp96 as well. With our approach, using VSV-specific CTL clones to detect the presence of viral peptides that endogenously associate with gp96, it is not possible to detect peptides that do not contain the core eight amino acid residues of VSV8, and this procedure is rather insensitive for the detection of the possibly longer precursor peptides of VSV8. In studies with synthetic peptides that are longer than the naturally produced peptides, sensitization of target cells requires peptide concentrations several orders of magnitude higher (unpublished observation). Most likely, these longer peptides are trimmed before they bind to MHC class I molecules in a manner that can be detected by VSV-specific TCRs. From the crystal structure of peptide/K^b complexes, it is clear that the limitation on the length of peptides that bind to MHC molecules is caused by a physical barrier of side chains from amino acid residues on the α-helices and β-sheet on both ends of the antigen binding groove (24, 25). Thus, it is unlikely that peptides longer than VSV8 bind to K^b on the target cells in our *in vitro* CTL test and, at the same time, expose TCR contact residues in the same orientation as VSV8. Despite the relative insensitive detection of longer (precursor) peptides, we did occasionally detect material in a HPLC fraction eluting at a higher acetonitrile concentration than VSV8, derived from

Table 2. H-2d class I molecules do not bind VSV8

	Specific lysis of EL4 target cells, %					
			Peptides stripped from the cell surface of			
Dilution	+VSV8*	−VSV8†	P815+VSV	P815−VSV	EL4+VSV	EL4−VSV
None	68	4	2	6	52	8
1:5	—	—	1	4	63	5
1:25	—	—	3	3	32	3

Peptides stripped from the surface of VSV-infected and uninfected EL4 cells (H-2b, 5×10^7) and P815 cells (H-2d, 10^8). Aliquots of peptides (25 μl, out of 1 ml, as well as 1/5 and 1/25 dilutions) were tested in triplicate for their ability to sensitize EL4 target cells (2000 per well) for recognition by VSV-specific CTL clone N15. Effector to target ratio = 5:1.
*Positive control, synthetic VSV8 (10 nM).
†Negative control, PBS.

gp96 molecules of VSV-infected EL4 and P815 cells. This fraction was 100-fold less potent in sensitizing the H-2b target cells, and not enough material was present to allow for further characterization (data not shown).

VSV8 is derived from the VSV nucleoprotein that is expressed in the cytosol of infected cells. Thus, viral peptides need to be transported into the ER before association with gp96 or K^b molecules can occur. The transporter associated with antigen processing (TAP) molecules in the ER membrane are the major supply of cytosolic peptides that bind to MHC class I molecules (26). This peptide transporter efficiently translocates peptides consisting of 9-16 amino acid residues (27). Thus, precursor peptides longer than the 8-9 amino acid residues that bind into the groove of MHC class I molecules get access to the ER. Recently, it has been shown that such longer precursor peptides can be trimmed in the ER to the size that efficiently binds the MHC class I antigen binding groove (28). The peptides that become available for binding gp96 are thus selected by the cytosolic proteolytic process and specificity of the peptide transporter complex TAP. Trimming in the ER may further contribute to the diversity in length of gp96-bound peptides.

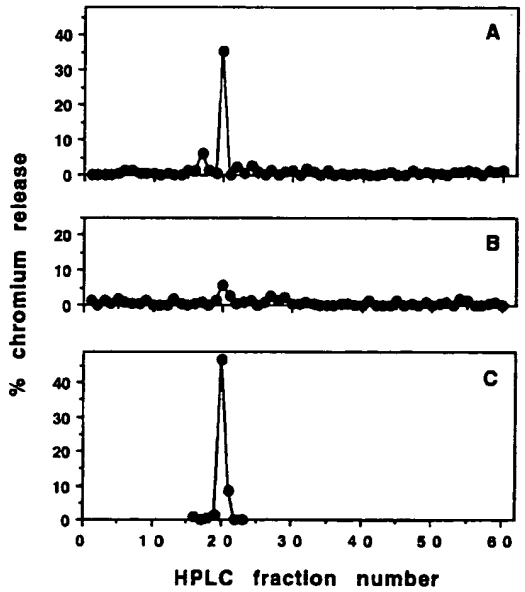

FIG. 4. VSV8 associates with gp96 in VSV-infected cells regardless of their MHC haplotype. (*A*) Peptides eluted from gp96 molecules purified from VSV-infected P815 cells (3×10^9, H-2d) sensitize H-2b target cells (EL4) for recognition by VSV-specific CTL clone N15. (*B*) No such activity is present in peptide mixtures isolated from uninfected P815 (3×10^9) cells. (*C*) The antigenic material eluted from gp96 (*A*) comigrates on HPLC with synthetic VSV8.

We have shown that the K^b-restricted antigenic peptide VSV8 can be isolated from P815 (H-2^d) cells that do not express H-$2K^b$ MHC class I molecules. This observation seems to be in contrast with reports from Rammensee and coworkers (29–31) in which they suggested that the cellular peptide content is governed by the MHC class I molecules expressed by a cell. However, their conclusion was based on experiments with only a limited number of antigenic peptides. Moreover, the same group recently published data showing that immunizations with gp96 isolated from H-2d cells results in cross-priming H-2b restricted minor H-specific CTL (32). They explain their results with the hypothesis that gp96 binds precursor peptides rather than finally processed peptides. Thus, in their earlier experiments, in which the cellular peptide composition was monitored with CTL clones, only the minimal epitopes were detected with high efficiency, underscoring the presence of precursor peptides.

This hypothesis may be true for some antigens. However, assuming that TAP transport and cytosolic processing determine the peptide pool that acquires acces to the ER, it seems reasonable to suggest that some peptides enter the ER as the minimal epitope that can bind to MHC class I molecules (with, as a possible example, VSV8), whereas others enter as precursor peptides that need further trimming before they can bind to MHC class I molecules. It is likely that both categories of peptides are candidates for association with gp96.

gp96 has been reported to efficiently shuttle associated antigenic material into the class I pathway of antigen presentation, resulting in the *in vivo* priming of T-cell responses against tumor- (13), viral- (33), and minor histocompatibility antigens (32). The growing list of antigens against which gp96 preparations can immunize suggests that gp96 forms a reservoir reflecting the cellular content of peptides that acquire access to the ER. In transformed cells or virus-infected cells, a subset of those peptides may be antigenic. Therefore, gp96 could function as a basis for vaccines that can be used in outbred populations against viruses and tumors with nonprivate antigens.

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